Characterization of the Helicoverpa Armigera Nucleopolyhedrovirus During Serial Passage in Cell Culture

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***Abstract*—*Helicoverpa armigera* nucleopolyhedrovirus (HearNPV) is highly pathogenic to the cotton bollworm, *H. armigera*. There have been several attempts to develop in vitro production of this virus, including the selection of high producing cell line. In order to evaluate the potential of in vitro methods of producing this virus on a large-scale, the virus was propagated in the *H. armigera* (HAPO2) and *H. zea* (HZ-AM1) cell lines and the correlation between the few polyhedra (FP) phenotype of HearNPV and the mutation in the *polyhedrin* (*polh*) and *fp25k* genes was analyzed. The many polyhedra (MP) phenotype was the predominant population of HearNPV for 3 passages in HZ-AM1 culture. The production of occlusion bodies (OBs) decreased with increasing passage number. The phenotypic observations on the HearNPV FP variants when this virus is serially passaged in HZ-AM1 cells suggest genomic changes occurring in the viral genes. A single point mutation resulting in an amino acid change was identified in the *fp25k* gene. This mutation could be correlated with the appearance of the FP phenotype with no relation to the *polh* gene. In contrast, serial passage in HAPO2 cells showed a slower accumulation of FP variants when compared to the use of HZ-AM1 cell line and no mutation was found in the *fp25k* gene. Therefore, HAPO2 cell line is a productive new cell line that will be useful for the scale-up process of HearNPV bioinsecticides.**

***Index Terms*—*Helicoverpa armigera*, *Helicoverpa zea*, nucleopolyhedrovirus, insect cell culture, few polyhedra, *fp25k gene*.**

I. Introduction

The cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae) is a major insect pest of a variety of crops grown in Thailand and world wide [1], causing destruction in produce quality and yield [2]. The most commonly practiced management strategy for this pest is the application of chemical insecticides. The application of chemical insecticides has generated concerns regarding the cost, development of resistance to the most commonly used insecticides, residues on crops and environmental pollution. Therefore, bioinsecticides form a challenging alternative. Especially nucleopolyhedroviruses (NPVs) are of interest, since they are highly specific for the target pest [3], and no resistance has been reported so far.

NPVs represent a group of insect virus in the Family Baculoviridae [4] whose virions are embedded into polyhedron-shaped occlusion bodies (OBs) or polyhedra in the nuclei of host cells. This OB facilitates virus survival and dispersal in the environment. *H. armigera* NPV (HearNPV) has been used to control *H. armigera* as an important part of the integrated pest management (IPM) program in Thailand [5]. However, the practical use of HearNPV in IPM program has been rather limited. One of the major problems has been that of production. The viral bioinsecticides can be produced in both in vivo and in vitro systems. The conventional method used to produce HearNPV for biological control purposes still employs the infection of large numbers of susceptible larvae, which is labor-intensive, variable potency, high production costs and it is difficult to scale up economically. The obvious alternative is the development of insect cell culture systems for in vitro production of viral agents. Growth and large-scale production of virus in insect cell culture has many attractive features that constitute clear advantages over in vivo production [6].

The establishment of new insect cell lines and of clonal cell lines with biotechnically desirable phenotypes, such as highly susceptible to the infection of NPV, has advanced markedly in the last decade [7], [8]. The successful replication of HearNPV in *H. armigera* (HA) and *H. zea* (HZ) cell lines has been reported [9]. Many works have been done to determine the replication of HearNPV in the HZ cell line which is commonly used for in vitro studies and production of HearNPV [10]-[15]. Serial passage of baculoviruses in cell culture caused a

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change from the many polyhedra (MP) to the few polyhedra (FP) phenotype. FP mutants are characterized by fewer cells containing polyhedra, fewer polyhedra per cell (contained less than 10 polyhedra per nucleus), polyhedra aberrant morphology and fewer or no viruses per polyhedron [16]. Serial passage of HearNPV in HZ cells leads to point mutations in the HearNPV *fp25k* gene, and the relationship between this mutation and the virulence of the virus is reported [14]. However, little is known about serial passage of HearNPV in HA cells.

In order to evaluate the potential of in vitro methods of producing this virus on a large-scale, the virus was propagated in HA and HZ cells and the correlation between the MP, FP phenotypes of HearNPV and the mutation in the polyhedrin (*polh*) gene that is responsible for polyhedron morphogenesis and a viral gene, *fp25k*, that encodes a protein involved in the FP phenotype of NPVs was analyzed.

II. Materials and Methods

A. Cell Line

The permissive cell line, KU-HAPO2, established from pupal ovaries of *H. armigera* at Central Laboratory and Greenhouse Complex, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Thailand [9] and BCIRL-HZ-AM1 cells [17] were grown as monolayers in 25-cm^2 tissue culture flasks. Cultures were maintained in Grace's medium [18] supplemented with 10% fetal bovine serum (FBS) and incubated at 27^{o}C. The culture medium was changed every 4 days.

B. Virus

HearNPV Prachin Buri isolate (collected from Prachin Buri Province, Thailand) was obtained from Prof. Tipvadee Attathom of the Department of Entomology, Faculty of Agriculture at Kamphaeng Saen. The virus was propagated in *H. armigera* larvae to generate infectious hemolymph containing budded viruses (BV) or extracellular virus (ECV). Fourth instar *H. armigera* larvae were fed on formalin-free artificial diet [19] that had been contaminated with HearNPV. Four days after inoculation, hemolymph containing ECV was collected by bleeding the infected larvae from the first proleg. The hemolymph was transferred immediately into ice-cold, sterile microcentrifuge tubes containing L-cysteine to prevent melanization [20], pooled and diluted 1:1 in culture medium. The diluted infectious hemolymph was passed through a 0.45 μm membrane filter and then purified by a plaque purification method [21] on HZ-AM1 cells. The MP variant of Thai HearNPV was designated HearNPV Prachin Buri1 (PCB1).

C. Serial Passage of HearNPV

The hemolymph of HearNPV PCB1-infected *H. armigera* larvae was used as passage zero (P0) for infection of the HAPO2 and HZ-AM1 cells to generate the ECV inoculum or the first passage (P1) of virus in cell culture. Cell monolayers were infected at a multiplicity of infection (MOI) of 1 plaque forming unit (PFU)/cell, the virus inoculum was allowed to adsorb for 1 h at room temperature with gentle rocking. At the end of the adsorption period the inoculum was removed and then the cells were rinsed with culture medium without FBS and maintained in complete medium at 27 ℃. The ECV from the culture medium of HAPO2 and HZ-AM1 cells 6 days postinfection (pi) was titrated by plaque assay [21].

The inoculum for the new passage (passage 2-6, P2-P6) was obtained by infecting cell monolayers with ECV from the previous passage at a MOI of 1 PFU/cell, the virus inoculum was allowed to adsorb for 1 h at room temperature and then replaced with fresh medium. Time zero was defined as the time when the inoculum was replaced with fresh medium. The medium containing ECV was harvested on day 6 pi, and determined the titer by plaque assay prior to use as inoculum for the next passage.

The yields of OBs derived from HearNPV-infected cells were determined at 6 days pi. Cells were lysed with 0.5% sodium dodecyl sulfate (SDS) for 30 min at room temperature, washed with sterile water and pelleted by centrifugation based on the protocol described by [22]. The yield of OBs from each treatment was counted using a Neubauer hemocytometer, with four replications per sample. OBs obtained from SDS-treated cells were also resuspended in a small volume of TE-8, and subsequent for viral DNA extraction.

D. Extraction of Viral DNA

The occlusion-derived virus (ODV) from the HearNPV-infected cells at each passage and ODV obtained from the infected larvae were used for DNA extraction and PCR amplification. To isolate the HearNPV DNA, 100 μl of OBs (10^7 OBs) of HearNPV in a microcentrifuge tube were mixed with an equal volume of 2X alkaline buffer (0.2 M Na_2CO_3, 0.02 M EDTA, 0.34 M NaCl) for 30 min on ice to dissolve OBs and release ODV. The undissolved OBs were separated by centrifugation at 12,000 rpm, for 1 min. The supernatant was incubated with proteinase K in the presence of SDS overnight at 37 ℃. After incubation with RNase A at 37 ℃ for 1 h, viral DNA was then purified with phenol/chloroform/isoamyl alcohol extraction followed by ethanol precipitation and was subsequently used for PCR amplification of the *polh* and *fp25k* genes.

E. PCR Amplification, Cloning and Sequencing of the polh and fp25k Genes

PCR was performed with specific primers designed from nucleotide sequence of HearNPV G4 *polh* and *fp25k* obtained from GenBank (accession number NC_002654) [23]. Two sets of primers were designed to amplify the entire regions including 5' and 3' flanking sequences of the HearNPV PCB1 *polh* and *fp25k* genes. Primers POLH-F (5'-GTG ATG AAC AAA ATA TGC GT-3') and POLH-R (5'-GCA GCG AAT TGA AAC AAT AT-3') were used to amplify a specific region of the *polh*, while primers FP-F (5'-GTA TTG TGT ACA CAC ACG TA-3') and FP-R (5'-TAT CAC AAC CAT ATG ACG GG-3') were used to amplify across the coding region of the *fp25k*, respectively. Additional primers were designed and used for primer walking sequencing to confirm the

nucleotide sequence of the first identified fragment of the gene, using the genomic HearNPV PCB1 DNA as template.

The PCR thermal profile consisted of an initial incubation at 94 ℃ for 5 min, followed by 35 cycles of 1 min at 94 ℃, 1 min at 56 ℃ and 1 min at 72 ℃, then a final extension at 72 ℃ for 5 min using a GeneAmp PCR System 9700, PE Applied Biosystems DNA thermal cycler. PCR amplification reaction volume was 50 µl which contained 1X PCR buffer (Invitrogen), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 U Taq DNA polymerase (Invitrogen), 5 pmol of each primer and 50 ng of template DNA.

The PCR products were analysed by agarose gel electrophoresis to determine the size, and the remaining products were either sequenced directly or cloned into pGEM®-T (Promega) and sequenced using the universal M13 forward and reverse primers with an Automate DNA sequencer (ABI PRISM 377, Applied Biosystems).

F. Electron Microscopy

Third instar larvae of *H. armigera* infected with HearNPV PCB1 at 3 days postinoculation were dissected and fixed with 3% glutaraldehyde in 0.1 M phosphate buffer pH 7.2, for 2 h at 4 ℃, postfixed in 1% osmium tetroxide for 1 h and were washed twice with phosphate buffer. After washing, the samples were sequentially dehydrated in acetone, infiltrated and embedded in Epon resin. Samples were cut with ultramicrotome, and observed under a transmission electron microscope.

III. Results and Discussion

A. Serial Passage of HearNPV

A newly plaque-purified MP variant of HearNPV, HearNPV PCB1, has a high insecticidal activity against the larvae of *H. armigera*. Electron microscopic observations confirmed that HearNPV PCB1 was a single-embedded NPV (Fig. 1).

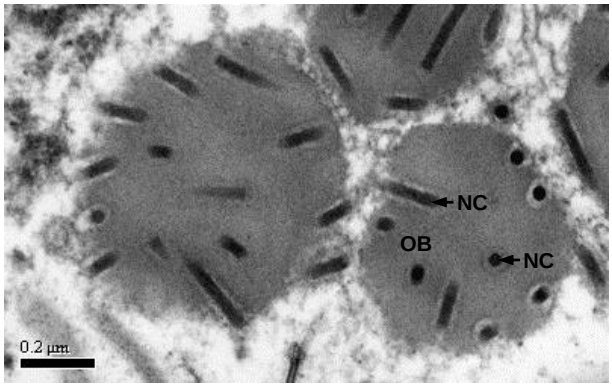

Figure 1. Electron micrograph of HearNPV PCB1 occlusion bodies (OBs) in infected cell of *H. armigera* larva. NC, nucleocapsid.

In order to investigate the effects caused by serial passage of HearNPV PCB1 in the HAPO2 and HZ-AM1 cell lines, the biological and molecular characteristics of the virus were analyzed until the infected cells appeared to contain the predominant population of FP variant. Upon infection with HearNPV PCB1, both HAPO2 (Fig. 2) and HZ-AM1 (Fig. 3) cell lines showed pronounced cytopathic effects (CPE) in which the nuclei of the cells were hypertrophied, and the first OB formation was observed by 1 day pi. At 6 day pi most of the cells became infected. Infected cells contained OBs and some of them detached from the culture flask.

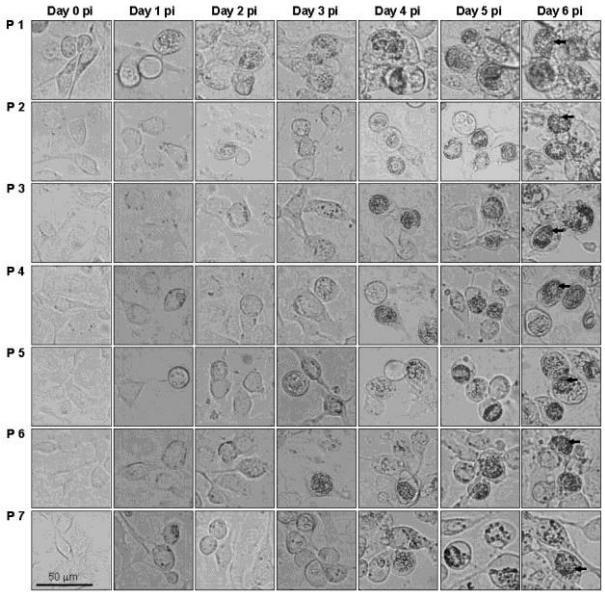

Figure 2. Cytopathic effects and OB formation of HearNPV PCB1 during serial passage in HAPO2 cells. OBs are indicated by arrows.

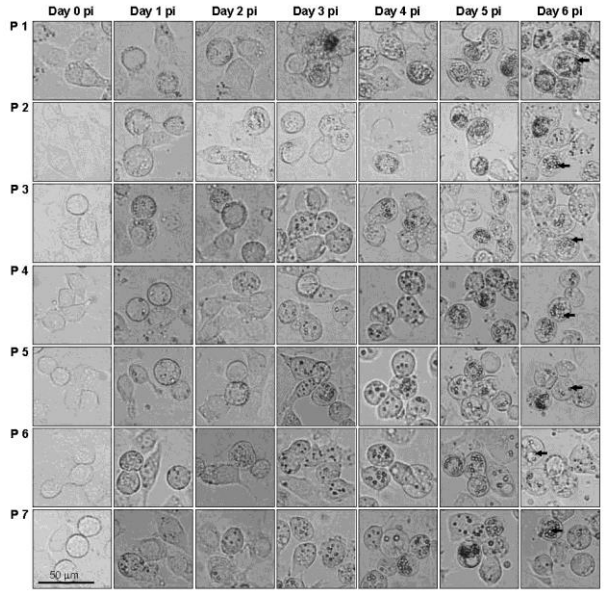

Figure 3. Cytopathic effects and OB formation of HearNPV PCB1 during serial passage in HZ-AM1 cells. OBs are indicated by arrows.

HearNPV PCB1 replicated well in the HAPO2 cells and exhibited the MP phenotype based on a large number of OBs produced per cell during serial passage. In contrast, the MP phenotype was the predominant population of the virus at passage 1 in HZ-AM1 culture after that, FP variants rapidly arose and became the predominant population during serial passage and transformed totally to a FP population at passage 7. In addition, morphologically abnormal OB was observed

when this virus was serially passaged in HZ-AM1 cells at passage 3 onwards (Fig. 4). The results demonstrated that HearNPV PCB1 decreased the total number of OBs produced and a few or a single or no OB was found in the nucleus. The virus also produced a few large cuboidal OB in the nucleus, some normal OBs were produced but the amount was greatly reduced.

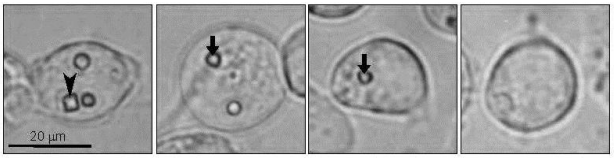

Figure 4. Abnormal OB production of HearNPV PCB1 in HZ-AM1 cells. The large cuboidal OB is indicated by arrowhead while the normal OB is indicated by arrow.

Further analyses of the virus were performed to study the kinetics of virus replication. The titer of ECV, amount of OB, and proportion of MP and FP plaques were determined for seven passages. The titer of ECV from HearNPV PCB1-infected HAPO2 cells was 1.7-fold greater at passage 7 compared to the titer produced at passage 2 (Fig. 5A). OB production was the very high level at passage 1 in the HearNPV PCB1-infected HAPO2 cells, decreased at passage 2 and remained steady until passage 4. OB amounts decreased in the HearNPV PCB1-infected HAPO2 cells from passage 5 to passage 7 and the number of OB produced per flask containing 2 x 10^6 cells decreased 2.5-fold from passage 1 to passage 7 (Fig. 6). In contrast, the titer of ECV from HearNPV PCB1-infected HZ-AM1 cells was 17-fold greater at passage 7 compared to the titer produced at passage 2 (Fig. 5B). The production of OB decreased with increasing passage number and the number of OB produced per flask containing 2 x 10^6 cells decreased 2.7-fold from passage 1 to passage 7 (Fig. 6).

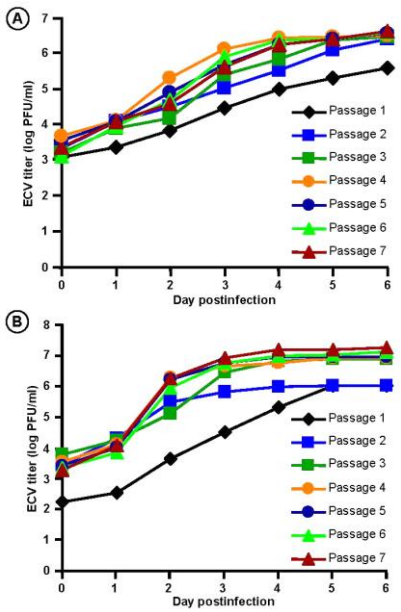

Figure 5. Production of ECV from the HearNPV-infected cells. The titer of virus was determined by plaque assay. (A) HAPO2 cell. (B) HZ-AM1 cell.

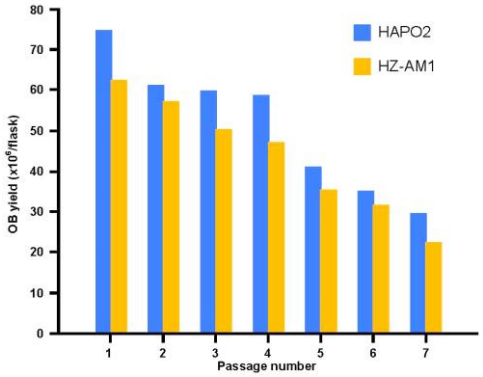

Figure 6. The yields of OBs derived from HearNPV-infected cells.

The percentage of HearNPV PCB1 exhibiting the FP or MP phenotype was determined during serial passage in the infected cells. ECV produced at each passage was used to generate viral plaques, the numbers of plaques were then used in calculating the percentage of MP and FP variants. The results in Fig. 7A revealed that the MP variant of HearNPV PCB1 was still the majority of the virus population at all passages throughout the experiment, while FP variant increased from 8% at passage 1 to 37% at passage 7 during serial passage in the HAPO2 cell. Unlike the HearNPV PCB1-infected HZ-AM1 cell, FP variant increased with a corresponding loss in number of the OB produced. The MP variant was the majority of the virus population for 3 passages in the infected HZ-AM1 cell and FP variant increased from 9% at passage 1 to 98% at passage 7 (Fig. 7B). Thus, the shift from the MP plaque variant to the FP variant appeared to be depended most critically on host cells and the number of virus passages.

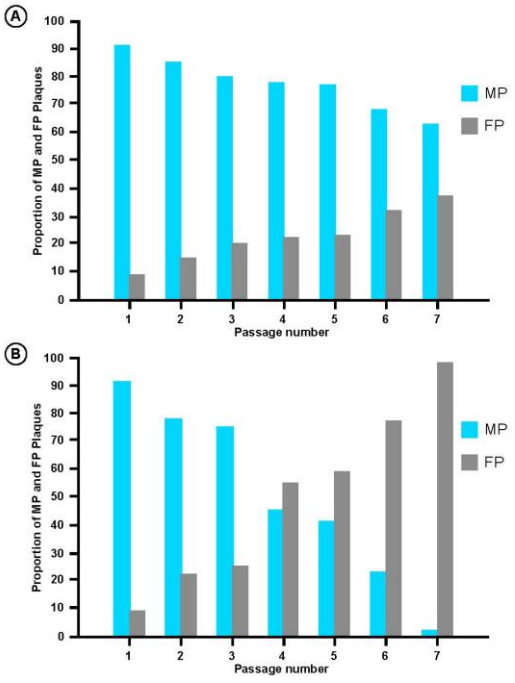

Figure 7. Proportion of MP and FP plaques obtained from ECV produced at each serial passage of HearNPV PCB1 in the infected cells. The plaques were visualized under an inverted microscope, MP and FP plaque phenotypes were enumerated. (A) HAPO2 cell. (B) HZ-AM1 cell.

B. *Molecular Characterization of HearNPV during Serial Passage in Cell Culture*

Since the HearNPV PCB1-infected HZ-AM1 cells showed typical signs of FP variant formation at passage 7, indicating that FP variant was becoming predominant in comparison to the MP variant at passage 1. The phenotypic observations on the HearNPV PCB1 FP variant when this virus is serially passaged in HZ-AM1 cells suggest genomic changes occurring in the *polh* and *fp* genes, which could be large insertions, deletions or substitutions within the gene. However, no significant alterations were found in the viral genome of passage 2 and passage 4 by restriction endonuclease (REN) analyses (Fig. 8). Therefore, serial passage of HearNPV PCB1 through the heterologous cell line, HZ-AM1, did not alter REN patterns of ODV.

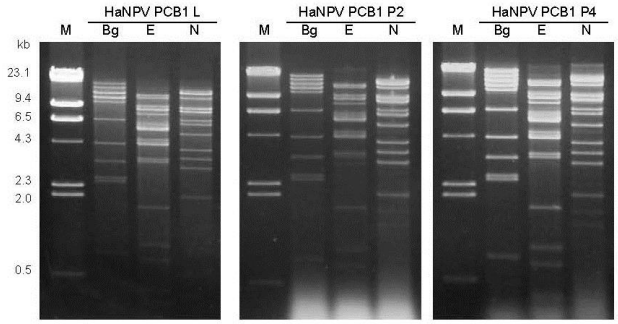

Figure 8. REN profiles of HearNPV PCB1. Genomic DNA derived from ODV produced at passage 2 (P2), passage 4 (P4) in the infected HZ-AM1 cells and DNA of ODV obtained from infected larvae (L) were digested with *Bgl*II (Bg), *Eco*RI (E), *Nco*I (N). The digested DNA was electrophoresed on 0.6% agarose gels along with λ DNA digested with *Hin*dIII (M).

To identify the *polh* gene of HearNPV PCB1 during serial passage in the HAPO2 and HZ-AM1 cells, a set of primers specific to the HearNPV *polh* gene designed from nucleotide sequence of HearNPV G4 *polh* [23] was used. The primers successfully amplified the entire regions including upstream and downstream flanking sequences, generated a 1029-bp PCR product (Fig. 9). PCR amplification of the genomic DNA derived from ODV produced at each serial passage of HearNPV PCB1 in the infected cells and DNA of ODV obtained from infected larvae generated a unique product of 1029 bp (Fig. 9). Nucleotide sequence analysis of the PCR product indicated the presence of an open reading frame (ORF) of 738 nucleotides which could encode 246 amino acid residues (Fig. 10) with a predicted molecular mass of 28.9 kDa. The nucleotide sequence of the HearNPV PCB1 *polh* revealed greater than 99% identity to that of the *polh* from other HearNPV isolates, including the two Chinese isolates, C1 (accession number NC_003094) [24] and G4 [23], two African isolates, NNg1 (accession number AP010907) [25] and South African (accession number AF157012) (unpublished), and Indian isolate PDBC (accession number FJ157293) (unpublished), while the predicted amino acid sequences of the *polh* ORF from these isolates were identical. Analyses of the nucleotide sequence of a 1029-bp PCR product obtained from HearNPV PCB1 as described above revealed that this fragment contained the *polh* gene and also demonstrated no mutation in the gene (Fig. 10).

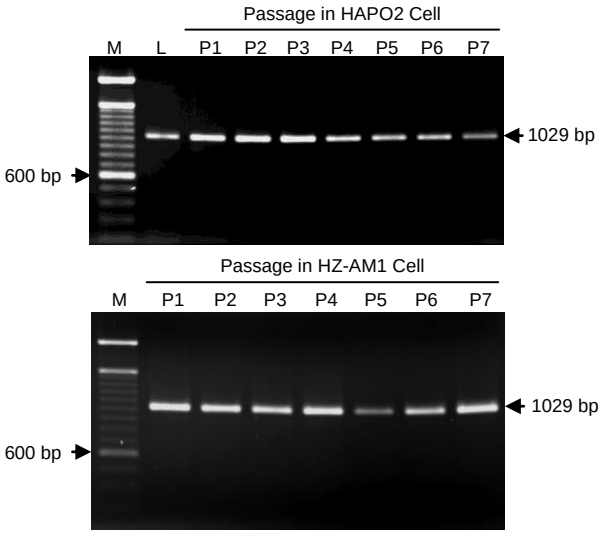

Figure 9. Agarose gel electrophoresis of PCR products amplified by using primer pairs specific to the HearNPV *polh* gene. Genomic DNA was isolated from ODV produced at each serial passage of HearNPV PCB1 in the infected cells (P1-7) and DNA of ODV obtained from infected larvae (L). The PCR products were electrophoresed on 1% agarose gels along with 100 bp DNA ladder (M). Position and size of the PCR products are shown on the right.

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POLH-L       MYTRYSYSPT LGKTYVYDNK YFKNLGAVIK NAKRKKHLEE HEHEERNLDS   50
POLH-P1-7HA  .......... .......... .......... .......... ..........   50
POLH-P1-7HZ  .......... .......... .......... .......... ..........   50

POLH-L       LDKYLVAEDP FLGPGKNQKL TLFKEIRSVK PDTMKLVVNW SGREFLRETW  100
POLH-P1-7HA  .......... .......... .......... .......... ..........  100
POLH-P1-7HZ  .......... .......... .......... .......... ..........  100

POLH-L       TRFMEDSFPI VNDQEIMDVF LSVNMRPTKP NRCYRFLAQH ALRCDPDYIP  150
POLH-P1-7HA  .......... .......... .......... .......... ..........  150
POLH-P1-7HZ  .......... .......... .......... .......... ..........  150

POLH-L       HEVIRIVEPS YVGSNNEYRI SLAKKYGGCP VMNLHAEYTN SFEDFITNVI  200
POLH-P1-7HA  .......... .......... .......... .......... ..........  200
POLH-P1-7HZ  .......... .......... .......... .......... ..........  200

POLH-L       WENFYKPIVY VGTDSAEEEE ILLEVSLIFK IKEFAPDAPL YTGPAY      246
POLH-P1-7HA  .......... .......... .......... .......... ......      246
POLH-P1-7HZ  .......... .......... .......... .......... ......      246
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Figure 10. Comparison of amino acid sequences of polyhedrin of HearNPV PCB1 produced in HAPO2 cell (P1-7HA), HZ-AM1 cell (P1-7HZ) and those of HearNPV PCB1 produced in *H. armigera* larvae (L). Dots indicate identical amino acids.

Genome alteration in HearNPV PCB1 was studied in detail by using a set of primers specific to the HearNPV *fp25k* gene designed from nucleotide sequence of HearNPV G4 *fp25k* [23]. The results indicated that primers could amplify the DNA derived from ODV produced at each serial passage of HearNPV PCB1 in the infected cells and DNA of ODV obtained from infected larvae, generated a reproducible amplification product of 745 bp (Fig. 11). Nucleotide sequence analysis of the 745-bp PCR product revealed an ORF of 651 nucleotides encoding 217 amino acid residues with a predicted molecular mass of 25.4 kDa, and this ORF is homologous to the *fp25k* of HearNPV C1 [24], G4 [23], NNg1 [25] and UQ-AC53 (accession number AF395841) [14]. Comparison of the deduced amino acid sequence of FP25K obtained from HearNPV PCB1 at each serial passage performed by using ClustalW is presented in Fig. 12. A single nucleotide substitution from A to G at position 4 of the gene resulting in an amino acid change of Lys2 to Glu was identified from passage 3 onwards.

Substitution at this residue resulted the amino acid at position 2 of HearNPV PCB1 FP25K to be identical to that of HearNPV C1 [24], G4 [23], NNg1 [25] and UQ-AC53 [14].

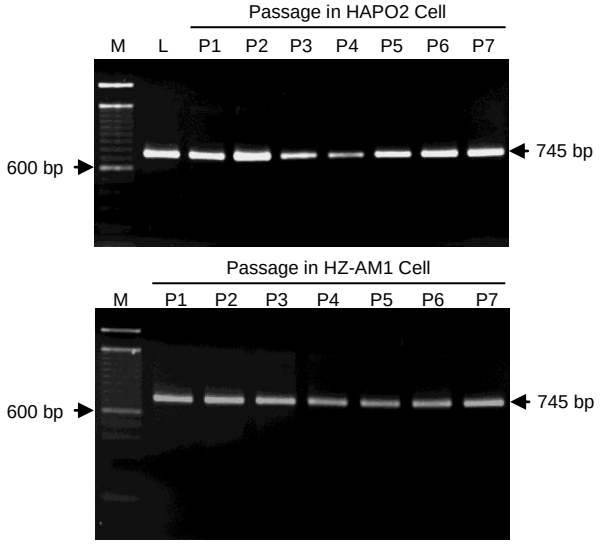

Figure 11. Agarose gel electrophoresis of PCR products amplified by using primer pairs specific to the HearNPV *fp25k* gene. Genomic DNA was isolated from ODV produced at each serial passage of HearNPV PCB1 in the infected cells (P1-7) and DNA of ODV obtained from infected larvae (L). The PCR products were electrophoresed on 1% agarose gels along with 100 bp DNA ladder (M). Position and size of the PCR products are shown on the right.

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FP-L        MKTDLINVPI LKSLIKHEID RSVNDNMSVI KGKIKKLEND KLNDTVEIYG   50
FP-P1-7HA   .......... .......... .......... .......... ..........   50
FP-P1-2HZ   .......... .......... .......... .......... ..........   50
FP-P3-7HZ   .E........ .......... .......... .......... ..........   50

FP-L        IHDRKLYNKK IRNNYVRKIC TLLDLDYRLV AETDFEKNHI CVKLSNAVTA  100
FP-P1-7HA   .......... .......... .......... .......... ..........  100
FP-P1-2HZ   .......... .......... .......... .......... ..........  100
FP-P3-7HZ   .......... .......... .......... .......... ..........  100

FP-L        KEWQTRSREV RLKNYDLDID YDGPVKIFVA ATAEHKQLLK KTRDALLPFY  150
FP-P1-7HA   .......... .......... .......... .......... ..........  150
FP-P1-2HZ   .......... .......... .......... .......... ..........  150
FP-P3-7HZ   .......... .......... .......... .......... ..........  150

FP-L        KYVSLCKRGV MVRRNDRSKV FIVKNELDIN ELVNKLYTKF DEENDKSTIA  200
FP-P1-7HA   .......... .......... .......... .......... ..........  200
FP-P1-2HZ   .......... .......... .......... .......... ..........  200
FP-P3-7HZ   .......... .......... .......... .......... ..........  200

FP-L        GIDSGIDEVD FVNTRLI                                      217
FP-P1-7HA   .......... .......                                      217
FP-P1-2HZ   .......... .......                                      217
FP-P3-7HZ   .......... .......                                      217
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Figure 12. Comparison of amino acid sequences of FP25K of HearNPV PCB1 produced in HAPO2 cell (P1-7HA), HZ-AM1 cell (P1-7HZ) and those of HearNPV PCB1 produced in *H. armigera* larvae (L). Dots indicate identical amino acids.

The present study provides more informations on the in vitro production of the HearNPV and susceptibility of its homologous cell line, HAPO2, and heterologous cell line, HZ-AM1. The results from the present study indicate that HearNPV FP variants rapidly arise and become predominant in the virus population during serial passage in the HZ-AM1 cells. The results from Fig. 5 also revealed that the progeny virus (P1) derived from inoculum P0 was a rather slow replicating virus, with CPE and OB formation occurring relatively late compared to the other passages. Perhaps this slow virus replication was due to melanization (blackening and precipitate formation), which tended to deteriorate the quality of the culture. Melanization in insects plays an important role in protection from virus infection [26]. This reaction is mediated by a cascade of serine proteases that cleave and activate prophenoloxidase that is then able to catalyze the oxidation of phenol to quinones [27]. It has been suggested that phenol oxidase may have an antiviral effect in the plasma of insects in addition to its role in capsulation [28]. Thus, melanization observed in insect cells inoculated with P0 inoculum may be responsible for the reduction in virus infectivity and the limitation of ECV production.

The biological characteristics of HearNPV PCB1 after passage the virus through the HZ-AM1 cells demonstrated it to be distinct from the original MP phenotype. The results revealed progeny virus having biological properties associated with FP phenotype, including FP plaque morphology, production of few or no OB although the nucleus was hypertrophied, high production of ECV and production of OB with abnormal morphology. Spontaneous mutation of MP variants together with the relationship between serial passage of NPV in cell culture and the appearance of FP mutant has been reported for several NPVs in different cell lines [10], [29]-[34]. In order to elucidate whether genetic alteration responsible for the HearNPV PCB1 FP phenotype, the *polh* and *fp25k* genes were analyzed. Comparison of the amino acid sequences of the polyhedrin reveals that the amino acid sequences are highly conserved among HearNPV, and shows no significant difference between HearNPV PCB1 produced at each serial passage and that obtained from the infected larvae. Interestingly, the amino acid substitution was found at the N-terminal sequences of FP25K from HearNPV PCB1 passage 3 onwards. The analysis of data from the present study and those of [16] and [35] indicate that FP25K mutants could cause increased production of ECV and decreased amount of ODV production with no relation to polyhedrin. In order to evaluate the FP mutation resulting from the insertion of transposon DNA into viral genome, the genomes of HearNPV PCB1 passage 2 and passage 4 were determined by restriction enzyme analysis. No insertion of transposable element originating from host cell DNA that could be correlated with the appearance of the FP phenotype [36] was detected and no significant alterations were found in the REN patterns of HearNPV PCB1. A similar result was observed with HearNPV DNA purified from ECV passage 2 to passage 6 that exhibited a FP mutant phenotype, no large insertion or deletion was indicated [14].

The FP mutant phenotype generally results from the insertion of host transposon DNA into the baculovirus genome. Several studies with *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) and *Galleria mellonella* MNPV (GmMNPV) have demonstrated that this insertion occurred in the *fp25k* gene at 5'-TTAA-3' tetranucleotide target sites [37]-[39]. The number of tetranucleotide target sites found in the *fp25k* gene of NPVs is variable. The 5'-TTAA-3' sites are present in 13 locations in the AcMNPV *fp25k* gene, while the homologs from HearNPV show 5-7 locations. The number of 5'-TTAA-3' target sites reported among the HearNPV *fp25k* gene, HearNPV PCB1 (sequence in the

present study), C1 [24], G4 [23] possessed 7 sites, HearNPV NNg1 possessed 6 sites [25], HearNPV ppC19 and UQ possessed 5 sites [14]. The results of this study are consistent with the previous report [14] that the FP mutation of HearNPV may arise through other mechanisms different from AcMNPV and GmMNPV [37], [38] but probably similar to those for LdMNPV [34, 40]. The data also support the conclusion that HearNPV FP mutants are not occurred via large DNA insertions or deletions, although 5'-TTAA-3' sites which are frequently associated with transposon insertions within the AcMNPV and GmMNPV *fp25k* genes were found. There is at present no studies that have demonstrated correlation between the greater number of 5'-TTAA-3' target sites identified in the AcMNPV *fp25k* gene and the higher rate of transposon insertions for this virus.

The rate at which FP mutations arise during serial propagation of NPVs in cell culture has been reported. The *Lymantria dispar* NPV (LdMNPV) FP mutants arise and become predominant very quickly during serial passage, greater than 92% of the virus exhibits a FP phenotype after the second passage [40]. The titer of ECV produced by LdMNPV is approximately 150- to 250-fold higher when the virus is passed serially for 3 passages, compared to that of ECV produced at the first passage. Similar results are obtained for *Trichoplusia ni* NPV (TnMNPV) [30]. FP plaques appear and displace production of MP virus after 8 consecutive passages, and FP virus persists homogeneous for 14 consecutive passages with no reversion to the MP phenotype. The results of the present study were in agreement with those obtained for LdMNPV and TnMNPV, ECV titer in both cell lines increased with increasing passage number. However, FP mutants accumulated more rapidly in HearNPV PCB1 passaged in HZ-AM1 cells than in those passaged in HAPO2 cells. In contrast to an increasing ECV titers of HearNPV PCB1, the results of previous studies [14] reported a increase in ECV titers from passage 3 to passage 4 during serial passage of HearNPV in HZ-AM1 suspension cultures followed by a decrease in virus titers from passage 5 onwards. A decrease or increase in ECV production of HearNPV during serial passage in cell culture is thought to depend on the virus-host interactions, since it has been reported that insect cell lines vary widely in their ability to support replication of HearNPV [9].

The results of the present study suggest that better OB yield of HearNPV PCB1 can be obtained by rigorous screening of available cell lines, whether they be heterologous or homologous. Therefore, homologous HAPO2 cell line is an important source of virus stability for the scale-up process of HearNPV bioinsecticides because serial passage in this cell line established from its natural host showed a slower accumulation of FP variants when compared to the use of the heterologous HZ-AM1 cell line.

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